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AQUATIC TOXICITY SCREENING OF AN ACWA SECONDARY WASTE, GB-HYDROLYSATE

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14. ABSTRACT: The Assembled Chemical Weapons Alternatives (ACWA) Program has been tasked to demonstrate alternative technologies to incineration that will safely dispose of assembled chemical munitions. The ACWA program is currently investigating GB-hydrolysate as a secondary waste that can be transported offsite to a commercial Treatment Storage and Disposal Facility (TSDF). The Microtox assay with bioluminescent marine bacterium <i>Vibrio fischeri</i> and the survival and reproduction of freshwater organism <i>Ceriodaphnia dubia</i> were used to investigate the aquatic toxicities of ACWA GB-hydrolysate or the neutralized (pH adjusted) ACWA GB-hydrolysate. The Microtox assay was also used to assess the aquatic toxicity of Bluegrass Depot GB-hydrolysate. The 5-min EC ₅₀ values for <i>V. fischeri</i> exposed to either ACWA GB-hydrolysate or neutralized ACWA GB-hydrolysate were 0.24 and 28% vol/vol, respectively. The 5-min EC ₅₀ values for <i>V. fischeri</i> exposed to Bluegrass GB-hydrolysate or neutralized ACWA GB-hydrolysate were 0.05 and 4.7% vol/vol, respectively. The 24-hr EC ₅₀ values for <i>C. dubia</i> exposed to either ACWA GB-hydrolysate or neutralized ACWA GB-hydrolysate were 0.8 and 1.3% vol/vol, respectively.					
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PREFACE

The work described in this report was authorized under Project No. 8VEJMH, Assembled Chemical Weapons Alternatives Program. The work was started in June 2008 and completed in September 2008.

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AQUATIC TOXICITY SCREENING OF AN ACWA SECONDARY WASTE, GB-HYDROLYSATE

1. INTRODUCTION

The Assembled Chemical Weapons Alternatives (ACWA) Program has been tasked to demonstrate alternative technologies to incineration that will safely dispose of assembled chemical munitions. The ACWA program is currently investigating GB-hydrolysate as a secondary waste that can be transported offsite to a commercial Treatment Storage and Disposal Facility (TSDF). Secondary waste must meet safety and environmental requirements and be approved by the regulatory agencies before offsite shipment can be considered. As part of the requirements, the toxicity of the waste must be characterized. The Environmental Toxicology Branch was tasked to conduct aquatic toxicity screening of GB-hydrolysate using the freshwater organism *Ceriodaphnia dubia* in a 7-day survival and reproduction assay and the bioluminescent marine bacterium *Vibrio fischeri* (NRRL B-11177) in the Microtox (MTX) assay. The neat ACWA GB-hydrolysate, as well as the neutralized (pH adjusted) ACWA GB-hydrolysate, were investigated. Samples of GB-hydrolysate from Bluegrass Army Depot (Richmond, KY) (Bluegrass) were also screened to toxicity using the MTX assay.

2. METHODS

2.1 GB-hydrolysate Production

Two GB-hydrolysate samples were produced by ACWA for investigation in this research project. The first sample, ACWA GB-hydrolysate (GB/NaOH GB-8072), was produced using 7.5% GB (Sarin, Chemical Agent Standard Analytical Reference Material, CASARM grade CAS#107-44-8; stabilized with tributylamine, CAS# 102-82-9) in 6% NaOH. The ACWA GB-hydrolysate was a clear golden brown color with very little precipitate. The second sample, Bluegrass GB-hydrolysate (040108-GB/NaOH), was produced from a GB Ton Container sampled in 2004 from Bluegrass. The Bluegrass GB-hydrolysate was produced using 7.3% GB (ton container sample) in 6% NaOH. The Bluegrass GB-hydrolysate was much darker in color and had noticeably more precipitate than the ACWA GB-hydrolysate. Portions of the ACWA and Bluegrass hydrolysate samples were separately neutralized (pH adjusted to 8.0 using 10% HCl) and used in toxicity testing (the Bluegrass GB-hydrolysates were screened using the MTX assay only). Due to the volume of 10% HCl needed to adjust the pH of the hydrolysate to 8.0, the final concentrations of the neutralized ACWA GB- and the Bluegrass GB-hydrolysates were 92.5 and 74.3%, respectively, of their original concentrations.

2.2 Microtox Assay

The MTX assay (Strategic Diagnostics Inc., Newark, DE) exposes bioluminescent marine bacteria (*V. fischeri*, NRRL B-11177), to a sample of unknown toxicity. The luminous flux from the bioluminescent bacteria is measured as the means of determining the level of toxic effects on the bacterial organisms. Under proper test conditions, reduction in light output is a

direct indication of metabolic inhibition. The bacteria were cultured by Strategic Diagnostics and shipped in lyophilized form. The bacteria (stored frozen) were re-hydrated immediately before testing. Individual assays were performed in a temperature-controlled photometer using glass cuvettes containing 1 mL of sample. For optimum accuracy in predicting toxicity the bioassay must include a minimum of four dilutions exhibiting a dose response, plus a control consisting of *V. fischeri* bioluminescent bacteria in MTX media. At 5 and 15 min, the control and treatment groups were measured for their respective luminous fluxes. Data were analyzed using the MTX 100% test protocol software to determine the EC₅₀ (the effective concentration causing a 50% reduction compared to light output by control).

A stock solution of 1% ACWA GB-hydrolysate was prepared using MTX test media, and serially diluted to obtain nominal treatment concentrations of 0.016, 0.031, 0.062, 0.125, 0.250, 0.500, and 1.0% vol/vol. Bluegrass GB-hydrolysate treatments were prepared using the same nominal concentrations. The neutralized ACWA GB-hydrolysate was added directly to MTX media and then serially diluted to produce nominal treatment concentrations of 0.72, 1.4, 2.9, 5.8, 11.6, 23.1, 46.2, and 92.5% vol/vol. The Bluegrass GB-hydrolysate was added directly to MTX media and then serially diluted to produce nominal concentrations of 1.2, 2.3, 4.6, 9.3, 18.6, 37.2, and 74.3% vol/vol.

2.3 Ceriodaphnia 7-Day Survival and Reproduction Assay

Ceriodaphnia 7-day Survival and Reproduction Assays were conducted with *C. dubia* according to the United States Environmental Protection Agency (USEPA) standard methods (1). The media for ceriodaphnia cultures consisted of 20% Perrier water and 80% reverse osmosis (RO) water. Ceriodaphnia were fed a mixture of green algae *Selenastrum capricornutum* (6×10^5 cells/mL) and cerophyl extract (20 µL/mL). Test chambers consisted of 30-mL plastic beakers containing a total of 15 mL of solution. Ten replicates of each treatment group and control (no GB-hydrolysate) were prepared, with each replicate containing one ceriodaphnia. The test media were renewed and fresh food added daily, for 7 d. Mortality, reproduction, pH, and dissolved oxygen measurements were recorded at 24 h intervals. A diurnal photoperiod cycle was maintained at 16 hr light:8 hr dark. The ambient light intensity was approximately 90 ft candles. The temperature was maintained at 25 °C. A stock solution of 1% ACWA GB-hydrolysate was prepared using test media, and serially diluted to obtain nominal treatment concentrations of 0.031, 0.062, 0.125, 0.25, and 0.5% vol/vol. A stock solution of 2% neutralized ACWA GB-hydrolysate was prepared using test media, and serially diluted to obtain nominal treatment concentrations of 0.031, 0.062, 0.125, 0.25, 0.50, and 1.0% vol/vol.

2.4 Determination of Toxicity Parameter Values and Statistics

Toxicity data were analyzed using regression models described in the Environment Canada Guidance Document (2). Having the best fit for data in the respective toxicity tests, either nonlinear logistic (Gompertz) model (eq 1) or linear model (eq 2) were used. The best fit of the lines generated by these models were closest to the data points, the variances of the residuals were the smallest, and the residuals had the best appearance (i.e., most random scattering). These models were

$$Y = a \times \exp[\log(1 - p)] \times (C \div ECp)^b \quad (1)$$

$$Y = [(-a \times p) \div ECp] \times C + a \quad (2)$$

where

Y	=	dependent variable (e.g., number of offspring)
a	=	the y-intercept (i.e., the control response)
exp	=	the exponent of the base of the natural logarithm
p	=	desired value for 'p' (e.g., 0.50 for EC ₅₀) inhibition
C	=	exposure concentration in test media
ECp	=	estimate of effect concentration for a specified percent effect
b	=	a scale parameter that defines the shape of the equation

The effective concentration parameters (ECp) used in this study included the nominal GB-hydrolysate concentration producing a 20% (IC₂₀) or 50% (IC₅₀) reduction in the measurement endpoint (i.e., luminous flux by *V. fischeri*; production of offspring by *C. dubia*) compared with control. Point estimation of EC₅₀ (the effective concentration that immobilizes 50% of adult *C. dubia*) calculations were performed using the Probit Analysis contained in the Minitab™ (MiniTab, State College, PA) statistical software package to assess adult survival. The 95% confidence intervals (CI) associated with the point estimates were also determined.

Analysis of Variance (ANOVA) was used to determine the bounded No Observed Effect Concentration (NOEC) and Lowest Observed Effect Concentration (LOEC) values for survival or reproduction data. Mean separations were determined using Fisher's Least Significant Difference (LSD) pair-wise comparison tests. A significance level of $p \leq 0.05$ was accepted for determining the NOEC and LOEC values. All analyses were done using nominal GB-hydrolysate concentrations. Statistical analyses were performed using SYSTAT® 11.0 (Systat Software Inc., Chicago, IL).

3. RESULTS

The MTX assays were conducted on ACWA GB- and Bluegrass GB-hydrolysates, as well as neutralized ACWA GB- and neutralized Bluegrass GB-hydrolysates. Overall, MTX results showed that Bluegrass-hydrolysate was approximately 4 times more toxic than the ACWA-hydrolysate, and the neutralized Bluegrass-hydrolysate was approximately 7 times more toxic than the neutralized ACWA-hydrolysate (Table 1).

Ceriodaphnia 7-day Survival and Reproduction Assays were conducted using the ACWA GB- and neutralized ACWA GB-hydrolysates (the Bluegrass GB-hydrolysate was not used in ceriodaphnia testing). The neutralized ACWA GB-hydrolysate showed approximately a 2 - 3 times reduction in short term (24 - 48 hr) acute toxicity when compared to the ACWA GB-hydrolysate (Table 2). The 7-day survival NOEC and LOEC values were also reduced with the neutralization of the ACWA GB-hydrolysate (Table 2). There were no differences in ceriodaphnia reproduction (NOEC/LOEC and EC₂₀/EC₅₀) between ACWA GB-hydrolysate and neutralized ACWA GB-hydrolysate (Table 2).

Table 1. Toxicity Benchmarks for GB-hydrolysate Determined by Microtox Assay Using the Marine Bacteria, *Vibrio fischeri*

Exposure Time	ACWA GB hydrolysate ^a EC ₅₀ % vol/vol (95% CI)	Neutralized ACWA GB hydrolysate (^a)EC ₅₀ % vol/vol (95% CI)	Bluegrass ^b GB hydrolysate EC ₅₀ %vol/vol (95% CI)	Neutralized Bluegrass ^b GB hydrolysate EC ₅₀ % vol/vol (95% CI)
5 min	0.24 (0.23-0.25)	29.0 (22.0-38.1)	0.05 (0.04-0.07)	4.56 (4.30-4.90)
15 min	0.22 (0.21-0.23)	25.1 (16.4-38.4)	0.06 (0.05-0.07)	3.20 (2.83-3.60)

^a ACWA GB-hydrolysate (GB/NaOH GB-8072) produced using 7.5% GB (CASARM grade) in 6% NaOH and neutralized with 10% HCl

^b Bluegrass GB-hydrolysate (040108-GB/NaOH) produced using 7.5% GB (ton container, sampled in 2004) in 6% NaOH and neutralized with 10% HCl

Table 2. ACWA GB-hydrolysate Toxicity to *Ceriodaphnia dubia*

Toxicity Benchmarks	ACWA GB-hydrolysate ^a % vol/vol (95% CI)	ACWA Neutralized GB-hydrolysate ^a % vol/vol (95% CI)
24-hr Acute EC ₅₀	0.81 (0.63 - 1.02)	1.30 (1.04-1.54)
48-hr Acute EC ₅₀	0.21 (CNBD) ^b	0.74 (CNBD) ^b
NOEC (Survival)	0.25	0.5
LOEC (Survival)	0.50	1.0
NOEC (Reproduction)	0.03	0.03
LOEC (Reproduction)	0.06	0.06
EC ₂₀ (Reproduction)	0.054 (0.045 - 0.063)	0.052 (0.044-0.060)
EC ₅₀ (Reproduction)	0.136 (0.112 - 0.159)	0.130 (0.110-0.149)

^a ACWA GB-hydrolysate (GB/NaOH GB-8072) produced using 7.5% GB (CASARM grade) in 6% NaOH and neutralized with 10% HCl

^b CNBD - could not be determined

4. DISCUSSION

The toxicities of GB-hydrolysates (ACWA and Bluegrass) to *V. fischeri* were reduced when the hydrolysates were neutralized, indicating that pH of the media could contribute to toxicity. Toxicity benchmarks for the effects of several hydrolysates (GD, GB, and HD, prepared for past programs) on *V. fischeri* are shown in Table 3 for comparison with the ACWA GB-hydrolysate. The GD-hydrolysate was produced using 2% GD (CASARM grade) in 18% NaOH, then neutralized with 10% HCl. The GB-hydrolysate was produced using 2% GB (CASARM grade) in 18% NaOH, neutralized with 10% HCl. The HD-hydrolysate was produced using 3.8% HD (ton container sample) hydrolyzed in water then neutralized with 0.5M NaOH. The toxicities of all these hydrolysates to *V. fischeri* were greater than either the neutralized

ACWA GB- or Bluegrass GB-hydrolysates, based on the EC₅₀ values listed in Table 3. The toxicity of the neutralized GB-hydrolysate listed in Table 3 was determined using an exposure medium that was prepared from 2% GB, yet it was 22 times more toxic than the neutralized ACWA GB-hydrolysate, which was prepared using 7.5% GB (3). The difference in the toxicity was likely due to the 18% NaOH used to produce that GB-hydrolysate. The resulting salt concentration (based on NaOH concentration) was 3 times greater compared with the salt concentration in the neutralized ACWA GB-hydrolysate; also, additional HCl was needed to neutralize 18% NaOH. The combination of high salt and additional HCl in that neutralized hydrolysate could cause greater osmotic stress, which contributes to its greater toxicity. The HD-hydrolysate listed in Table 3 was produced from ton container samples (4). When compared to the neutralized ACWA GB and Bluegrass GB-hydrolysate, the HD-hydrolysate was approximately 50 and 8 times more toxic to *V. fischeri*, respectively.

Table 3. Toxicities of Neutralized Hydrolysates to Bioluminescent Marine Bacterium, *Vibrio fischeri*

Exposure Time	GD-hydrolysate ^a EC ₅₀ % vol/vol	GB-hydrolysate ^b EC ₅₀ % vol/vol	HD-hydrolysate ^c EC ₅₀ % vol/vol	ACWA GB-hydrolysate ^d EC ₅₀ %vol/vol
5 min	1.1	1.3	0.58	29.0

^a Hydrolysate produced using 2% GD (CASARM) in 18% NaOH, neutralized with 10% HCl. (3)

^b Hydrolysate produced using 2% GB (CASARM) in 18% NaOH, neutralized with 10% HCl. (3)

^c Hydrolysate produced using 3.8% HD (Ton Container) in water, neutralized with 0.5M NaOH. (4)

^d Hydrolysate produced using 7.5% GB (CASARM) in 6% NaOH, neutralized with 10% HCl.

The Ceriodaphnia 7-Day Survival and Reproduction Assay followed USEPA method EPA-821-R-02-013. These methods are used by USEPA state and regional offices, as well as National Pollutant Discharge Elimination System (NPDES) permittees. All wastewater treatment facilities that discharge into waters of the United States are required to conduct Ceriodaphnia 7-Day Survival and Reproduction Assay annually and provide data showing they are meeting discharge criteria. The ceriodaphnia data generated from this project will help regulators determine if it is feasible for the secondary ACWA waste (GB-hydrolysate) to be permitted for disposal using a Waste Water Treatment Facility (WWTF) or a TSDF.

The toxicity results were ranked using the Chemical Scoring System for Hazard and Exposure Identification (5). This system is typically used in the preliminary screening process and is not intended to be a substitute for risk assessment. This system assigns a score (0-9; 9 being the most toxic) based on the acute toxicity data and chronic (NOEC) toxicity data. The units of the toxicity values presented used in this system are milligrams/liter. Therefore, the toxicity data generated from this study were transformed from percent volume/volume to milligrams/liter on the basis of our density determination measurements and calculations. The scoring system developed by O'Bryan and Ross (5) does not rank the scores using common terms typically used in mammalian toxicity rankings; however, the U.S. Fish and Wildlife Service (USFWS) published a Research Information Bulletin (6) suggesting relative aquatic toxicity terms (ranks) that we used based on EC₅₀ data. The ranking system considers EC₅₀

results > 1000 mg/L to be “Relatively Harmless” and results < 0.01 mg/L as “Super Toxic.” Similar descriptive rankings for mammalian toxicity are used by Kamrin (7).

The scoring protocols stipulate that when multiple scores are assigned in the acute and chronic category, the highest score (most toxic) should be selected as the overall aquatic toxicity score. Using the Chemical Scoring System for Hazard and Exposure Identification, the ACWA GB-hydrolysate and Neutralized ACWA GB-hydrolysate scored a 0, which ranked these hydrolysates as Relatively Harmless (Table 4). The Bluegrass GB-hydrolysate scored a 2 based on *V. fischeri* data and was ranked as Practically Non-Toxic. For comparison, acetone was ranked using data from *V. fischeri* (Microtox) and *C. dubia* assays (8), and malathion was ranked using *C. dubia* assay. Acetone scored 0, which is ranked as “Relatively Harmless” (similar in toxicity to GB-hydrolysate). For comparison, the 48-hr *C. dubia* EC₅₀ for malathion was approximately 0.002 mg/L (9,10), which scored a 9 and ranked malathion as “Super Toxic.”

5. CONCLUSIONS

- The Assembled Chemical Weapons Alternatives (ACWA) GB-hydrolysate was less toxic to *V. fischeri* than Bluegrass GB-hydrolysate.
- Neutralization decreased the toxicities to *Vibrio fischeri* of the ACWA GB- and Bluegrass GB-hydrolysates.
- The neutralized ACWA GB-hydrolysate was less toxic to ceriodaphnia than the ACWA GB-hydrolysate, based on the acute toxicity data.
- The neutralized ACWA GB-hydrolysate was similar in toxicity to ACWA GB-hydrolysate when compared on the basis of ceriodaphnia reproduction toxicity data.
- Overall, the ACWA GB-hydrolysate and the neutralized ACWA GB-hydrolysate received toxicity rankings of Relatively Harmless [based on ceriodaphnia and Microtox (MTX) data].
- The Bluegrass GB-hydrolysate received a toxicity ranking of Practically Non-Toxic (based on MTX data).

Table 4. Toxicity Comparison for GB-hydrolysates, Acetone, and Malathion Using O'Bryan and Ross Chemical Scoring System for Hazard and Exposure Identification (5) and the U.S. Fish and Wildlife Service Ranking (6).

Toxicity Benchmarks	Concentrations (mg/L)	Scores (1-9, 9 being most toxic)	Rankings
ACWA GB-hydrolysate			
<i>V. fischeri</i> 5-min EC ₅₀	2450	0	Relatively Harmless
<i>C. dubia</i> 24-hr EC ₅₀	7330	0	Relatively Harmless
<i>C. dubia</i> NOEC, 7-D	328	0	Relatively Harmless
Neutralized ACWA GB-hydrolysate			
<i>V. fischeri</i> 5-min EC ₅₀	327,100	0	Relatively Harmless
<i>C. dubia</i> 24-hr EC ₅₀	14,518	0	Relatively Harmless
<i>C. dubia</i> NOEC, 7D	351	0	Relatively Harmless
Bluegrass GB-hydrolysate			
<i>V. fischeri</i> 5-min EC ₅₀	576	2	Practically Non-Toxic
Neutralized Bluegrass GB-hydrolysate			
<i>V. fischeri</i> 5-min EC ₅₀	870	2	Practically Non-Toxic
Acetone			
<i>V. fischeri</i> 5-min EC ₅₀	18,170	0	Relatively Harmless
<i>C. dubia</i> 96-hr EC ₅₀	8,098 ^a	0	Relatively Harmless
Malathion			
<i>C. dubia</i> 96-hr EC ₅₀	0.002 ^b	9	Super Toxic

^a Cowgill and Milazzo, 1991

^b Ankley et al., 1991, and , Maas, 1982

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